Persistence of anti-zonae pellucidae antibodies following a single inoculation of porcine zonae pellucidae in the domestic equine

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Abstract

In this study of equids, we investigated the antibody response and the effect on the estrous cycle following a single inoculation of porcine zonae pellucidae (pZP) employing controlled-release methodology. We also investigated the use of two different water-soluble adjuvants as an alternative to oil-based adjuvants. Twenty-seven domestic mares were inoculated with various formulations of pZP and adjuvant. We showed that the anti-pZP antibodies generated as a result of the inoculations persisted for at least 43 weeks (length of the study). Of the various formulations used in the study, pZP and QS-21 water-soluble adjuvant, administered in combination with an emulsified preparation of pZP and Freund's Complete Adjuvant generated a significantly ($P < 0.05$) higher titer of anti-pZP antibodies when compared with other formulations employing the water-soluble adjuvant, Carbopol. Hormone analyses for cyclicity indicated a high incidence and extended duration of persistent corpora lutea among the treated mares. The positive control group of mares receiving two standard inoculations of pZP and Freund's Complete and Incomplete Adjuvants, as well as the placebo group of mares injected with QS-21 only, also exhibited high incidences of persistent corpora lutea. However, all mares eventually returned to normal cyclicity. The basis for the high incidence and extended duration of persistent corpora lutea was unexplained. The results demonstrate for the first time the persistent generation of anti-pZP antibodies following a single inoculation of pZP incorporated into a controlled-released preparation in the horse. This study further suggests that a single inoculation of pZP sequestered in a controlled-release lactide-glycolide polymer may serve as an alternative to traditional two-inoculation protocols for contraception investigations in the equine.

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Introduction

The use of porcine zonae pellucidae (pZP) as a contraceptive agent in female horses has been reported (Liu *et al.* 1989, Kirkpatrick *et al.* 1990, 1992, Willis *et al.* 1994, Turner *et al.* 1997, 2001). Inoculation of the heterologous antigen into horses develops systemic, humoral antibodies that are effective in preventing pregnancy in mares. It is suggested that blockage of fertility is achieved by sperm receptor binding by the antibody or by steric hindrance (Liu *et al.* 1989). These studies further demonstrated no disturbances in cyclicity or behavioral patterns of the inoculated females; treatment of pregnant mares yielded no untoward effects on their pregnancies or resultant foals

and no adverse effects on the ovaries were noted based on ovarian pathology. Furthermore, contraception was reversible when the antibody titer decreased to 50–60% of the positive reference sera.

When pZP is used as a contraceptive agent, at least two inoculations, administered 4 weeks apart are required. The first inoculation initiates antigen recognition while the second inoculation serves as a booster (Liu *et al.* 1989, Kirkpatrick *et al.* 1991). The effectiveness of the vaccine appears to be dependent upon the level and duration of the anti-zona antibodies generated as a result of a series of at least two inoculations. In one study (Liu *et al.* 1989), anti-pZP antibodies generated from four to six inoculations comprising 65 μg pZP, combined with either Freund's

Complete Adjuvant (FCA) or Incomplete adjuvant (FIA) and administered 4 weeks apart, persisted over a period of 21–40 months. The contraceptive effect lasted for a period of at least 8 months in the presence of these antibodies. In another study (Willis *et al.* 1994), using two inoculations of pZP and a synthetic adjuvant, trehalose dicorynomycolate glycolipid, incorporated into a calcium carbonate and hydoxypropylcellulose Biobullet, anti-pZP antibody persisted for at least 40 weeks. Although these previous studies demonstrate that anti-pZP antibodies are generated as a result of a series of inoculations with pZP, characterization of the antibody response following a single inoculation of pZP has not been defined. These reports, however, strongly suggest that pZP may serve as an attractive alternative for limiting pregnancy in mares. Additionally, pZP may be effective in limiting the number of horses in areas where overpopulation is a problem and where culling or decreasing the size of horse herds by euthanasia, translocation and/or slaughter is impracticable or prohibited.

Successful field applications of pZP vaccines in feral horse populations have been reported (Kirkpatrick *et al.* 1990, 1992, Turner *et al.* 2001). The impracticability of administering two inoculations by hand injection 4 weeks apart is especially apparent when feral horse populations are targeted. This method of administration imposes additional stress to horses during the 4 week captivity period, individual identification of the animal is mandatory and the maintenance of these animals during the holding period requires additional personnel time and expense. When non-captive procedures are utilized, such as in remote delivery systems, locating previously inoculated horses is difficult and approaching a previously inoculated and wary feral animal to administer the second inoculation is impracticable. These factors make the use of a single-inoculation vaccine attractive for regulating pregnancy rates in large populations of feral horses.

Controlled-release technology employing poly(lactide-co-glycolide) (PLGA) biodegradable polymers has been used for the delivery of a number of therapeutic agents including anti-cancer agents and various vaccines (Laemmli 1970, Chang 1976, Carter *et al.* 1988, Wang *et al.* 1990, 1991). This technology may be applied to the pZP antigen to meet the requirements of a single-injection vaccine needed for remote treatment of wildlife by

providing release of the required second exposure by controlled-release methods. Using this technology, pZP antigen and/or adjuvant can be sequestered within inert, nontoxic PLGA copolymer pellets which would exhibit delayed release of their contents upon injection and exposure to biological fluids in a fashion dependent on the copolymer ratio, preparation method and vaccine/adjuvant loading.

The purpose of this study was to investigate controlled-release technology as a means of achieving long-lasting antibody responses via a single inoculation. This study also attempted to characterize the antibody response and cyclicity of domestic mares as a result of the inoculation, through frequent blood sampling intervals. In addition, a suitable water-soluble adjuvant that would serve as an alternative to oil-based adjuvant for incorporation into controlled-release formulations was investigated.

Materials and Methods

Animals

Twenty-seven domestic mares of varying breeds were used in this study. The mares ranged from 5 to 15 years of age, were known to be cycling and were in healthy condition. The reproductive tracts of the mares were examined by palpation per rectum, ultrasonography and speculum examination for gross abnormalities. A complete reproductive soundness examination was not performed since fertility was not an end point of this study. The mares were divided into five groups of five mares each and one group of two mares. Each group of mares was administered different formulations of pZP and adjuvants as described in Table 1. Group 1 mares ($n = 5$), were inoculated with a single injection of 413 µg pZP + FCA, one controlled-release pellet comprising 200 µg pZP and one controlled-release pellet containing 250 µg QS-21 adjuvant (Aquila Pharmaceuticals, Framingham, MA, USA). Group 2 mares ($n = 5$) were inoculated with a single injection of 400 µg pZP + FCA, one blank pellet and six pellets containing 7 mg Carbopol 974 adjuvant (B F Goodrich, Cleveland, OH, USA). Group 3 mares ($n = 5$), were inoculated with a single injection containing 413 µg pZP+FCA, one pellet containing 200 µg pZP and six pellets containing Carbopol 974 adjuvant (7 mg) in

Table 1 Experimental groups and formulations of pZP used in each group. Formulations for each group were combined as a single inoculation except Group 6 in which two inoculations were administered 4 weeks apart.

Group	No. of mares	Emulsified antigen	Pelletized booster antigen	Pelletized adjuvant	No. of inoculations
1	5	400 µg pZP+FCA	200 µg pZP	250 µg QS-21	1
2	5	400 µg pZP+FCA	Blank	7 mg Carbopol	1
3	5	400 µg pZP+FCA	200 µg pZP	7 mg Carbopol	1
4	5	65 µg pZP+FCA	200 µg pZP	7 mg Carbopol	1
5 (control)	5	1 ml PBS	—	250 µg QS-21	1
6	2	(1) 65 µg pZP+FCA (2) 65 µg pZP+FIA	—	—	2

each pellet. Group 4 mares ($n = 5$), were inoculated with a single injection of 65 µg pZP + FCA, one pellet containing 200 µg pZP and six pellets of Carbopol adjuvant. All pZP used in the controlled-release copolymer pellets was in lyophilized form. Emulsified pZP + FCA, pelleted pZP and adjuvants used in each group (Groups 1–4) were combined as a single injection. The pellets were inserted into a 14 gauge, 1-1/2 inch hypodermic needle, attached to a 5 ml plastic syringe containing 1.0 ml emulsified pZP + FCA and administered into the semimembranosis and semitendinosis muscles of the left hind leg. Group 5 mares were inoculated with a single injection comprising 1 ml PBS and one pellet containing 250 µg QS-21 adjuvant. Group 6 mares ($n = 2$) served as a reference point for a standard two-inoculation protocol. They were inoculated with two injections into the semimembranosis and semitendinosis muscles, with 65 µg pZP + FCA (emulsified as a solution) administered as the initial injection, and 65 µg pZP + FIA given 4 weeks later as a second injection.

Blood samples obtained from the jugular vein of all mares were collected at the time of inoculation and every week for 44 weeks. Sera were separated from the samples and stored at 4 °C until used for anti-pZP antibody and hormone determinations.

All procedures involving animals were performed in accordance with the guidelines of the Office of Environmental Health and Safety and under the Protocol for Animal Use and Care (protocol #8394), University of California, Davis, CA.

Preparation of the zona antigen

Heterologous zonae pellucidae antigen was prepared using porcine oocytes as described by Liu *et al.* (1989). Briefly, frozen–thawed ovaries were sliced in a ganged razor blade apparatus. The oocytes were separated through a series of meshed screens of 210, 150 and 73 µm in the presence of PBS (pH 7.2). The oocytes were then homogenized, trapped again on a 48 µm meshed screen, rinsed with PBS and heat solubilized at 70 °C for 50 min in PBS for inoculation doses and 0.1 M glycine buffer (adjusted to pH 9.5 with NaOH) when used for ELISA. Protein determination was performed using the Automated Protein Microassay (Bio-Rad Laboratories, Richmond, CA, USA) procedure using BSA as the standard.

Determination of the pZP glycoprotein specificity of mare serum

pZP (12.5 µg total at 1 µg/ul) was mixed with 112.5 µl rehydration buffer containing 2.2 M thiourea, 7.7 M urea, 4.4% 3-cyclohexylamino-1-propensulfonic acid, immobilized pH gradient (IPG) buffer pH 3–10 (final concentration 1% v/v) (Amersham Pharmacia), 0.55% Triton X-100 and 10.1 mg/ml dithiothreitol (DTT). The first-dimension isoelectric focusing (IEF) separation was

performed using an Amersham Pharmacia Ettan IPGphor following the manufacturer's instruction. The IPG strips (7 cm) were used for the first-dimension separation. After use, the strips were stored at −20 °C for the second-dimension separation.

The room temperature IPG strip was subsequently soaked in equilibration buffer containing 20 mg/ml DTT with gentle inversion for 15 min at room temperature, transferred to a new tube and soaked in equilibration buffer containing 25 mg/ml iodoacetamide for 15 min. The second-dimension SDS-PAGE separation was performed according to the method of Laemmli (1970) using an 8% gel. The IPG strip was held in place on top of the stacking gel with 1% agarose in equilibration buffer.

Silver staining of the gel

After the SDS-PAGE, the gel was fixed with 25% methanol–5% acetic acid for 20 min. The gel was immersed in 50% methanol solution for 20 min, and then washed with deionized water for 10 min. Silver staining of the gel utilized the method of Shevchenko *et al.* (1996). Digital images of the stained glycoproteins patterns were immediately captured; the gel was subsequently stored in 1% acetic acid solution.

Western blots

The two-dimensional PAGE-separated glycoproteins were transferred onto PVDF membrane (Immobilon-P; Millipore) as per Murata *et al.* (1993). After transfer, the membrane was rinsed with Tris-buffered saline (50 mM Tris–150 mM NaCl), pH 7.4, containing 0.05% Tween 20 for 15 min. The rinsing process was repeated three times. The primary antiserum for the single horse serum was diluted 1:100 in Tris–saline–Tween solution containing 2% BSA; for pooled sera, the dilution was 1:20 000. The membrane was incubated in the primary antibody solution at 4 °C overnight. After the incubation, the membrane was rinsed with Tris–saline–Tween solution as above and then incubated in Tris–saline–Tween–BSA solution containing rabbit anti-goat IgG-conjugated horseradish peroxidase (single serum, 1:100; pooled sera, 1:10 000) at room temperature for 2 h. After washing, as described above, the IgG-conjugated horseradish peroxidase was localized using a TMB substrate kit (Vector Laboratories, Burlingame, CA, USA) as per the manufacturer's instruction. Images of the stained gels were immediately captured.

Immunohistochemistry: specificity of anti-pZP antibodies

Representative follicles from ovaries (horse) and ovaries from bitches were fixed in 4% paraformaldehyde at 4 °C for 24 h and processed as previously described (Conley *et al.* 1995). Immunocytochemical localization of anti-pZP antibodies was performed using the avidin-biotin-peroxidase complex method with a Vectastain Elite ABC kit

(Vector Laboratories). Sections of dog and horse tissue were deparaffinized using CitriSolv (Fisher Health Care, Houston, TX, USA), hydrated through a graded alcohol series and rinsed in water. Endogenous peroxidase activity was then blocked in 0.3% H_2O_2 for 30 min followed by washing in buffer. Sections were blocked in dilute normal goat serum (KPL, Gaithersburg, MD, USA) for 20 min, washed in PBS, incubated with avidin and biotin blocking solutions with PBS washes in between, then incubated overnight at 4 °C with the primary antibody (1:1500) obtained from a pool of treated horses. Negative control sections were treated similarly and incubated with serum from untreated horses (1:1500). Dilute biotinylated goat anti-horse IgG secondary antibody was applied for 30 min followed by a 30 min incubation with the avidn-biotin-peroxidase complex. Visualization was achieved using a Vector Nova Red Substrate kit (Vector Laboratories). Slides were counterstained with hematoxylin, dehydrated through a graded alcohol series, submerged in Citrisol for 4 min and mounted using Permount mounting media (Fisher Scientific, Tustin, CA, USA).

Pellet fabrication

pZP was first lyophilized from an aqueous solution to obtain a solid that could be mixed with the PLGA copolymer. The copolymer of lactide:glycolide (65:35) was obtained from Birmingham Polymers, Inc. (Birmingham, AL, USA). It was spray-dried from a methylene chloride solution to obtain a fine powder of the copolymer. Lyophilized pZP and spray-dried PLGA copolymer were mixed with an agate mortar and pestle to obtain a 1% w/w mixture of pZP in PLGA. This mixture was compressed into pellets with a 1.6 mm punch and die at a total force of about 100 lbs for 60–90 s on a QTest mechanical tester (MTS Systems Corp., Eden Prairie, MN, USA). This process generated pellets that were about 10 mg in weight containing 90–200 μg pZP and 5 mm in length by 1.5–1.6 mm in diameter. The pellets could easily fit into a 14-gauge needle for injection. Adjuvant (Carbopol or QS-21) was incorporated into pellets in a similar fashion in which the powdered adjuvant was blended with the spray-dried copolymer and compressed into 10 mg pellets of the same dimensions as the pZP pellets. The Carbopol content was 1.1–1.2 mg/pellet and the content of QS-21 was 250 μg/pellet. The length of delay in pZP or adjuvant release was adjusted by using copolymer of differing lactide:glycolide ratio. A lactide:glycolide copolymer ratio of 65:35 ratio was expected to release pZP in 3–4 weeks.

Enzyme immunoassay for progesterone

Luteal response was determined by progesterone analysis of plasma by an enzyme immunoassay as described by Munro & Stabenfeldt (1984). Progesterone 3-O-carboxy-methyloxime–horseradish peroxidase was used as the label and antiserum from rabbits was raised against a progesterone 11-alpha-hemisuccinyl-BSA immunogen. Progesterone 3-O-carboxy-methyloxime and 11-alpha-hemisuccinyl-BSA were purchased from Steraloids, Inc. (Wilton, NH, USA). Horseradish peroxidase was obtained from Sigma Chemical Co., (St Louis, MO, USA). The mean intra-assay coefficient of variation was 9.97% and the average interassay coefficients of variation for pools of high, medium and low progesterone concentration were 4.9, 6.2 and 10.5% respectively. The sensitivity of the progesterone assay was < 0.1 ng/ml. Progesterone was measured for each mare as a single assay. Samples were run in duplicates with pooled mare sera with known levels of progesterone as controls for every 20 samples.

ELISA for the determination of anti-pZP antibodies

The assay was performed as previously described by Liu et al. (1989). Briefly, 50 μl of a 5 μg/ml pZP antigen solution in 0.1 M glycine buffer, pH 9.5, was placed in each well of a flat-bottom Microelisa Immulon 2 plate (Dynatech Laboratories, Alexandria, VA, USA) and incubated overnight at 4 °C. The plate was washed once and incubated with 200 μl PBS–Tween for 30 min to block unspecific binding sites. After two further washes (PBS–Tween), the treatment of the plate consisted of subsequent 1 h incubations with 50 μl/well of PBS–Tween-diluted reagents. The reagents were used in the following order with three washes each in between: (i) test serum 1:1000, (ii) biotinylated goat anti-horse IgG (Zymed Laboratories, San Francisco, CA, USA) 1:1000, and (iii) bioavidin (Zymed) 1:2000. Finally, 50 μl substrate solution of 1 mg p-nitrophenyl phosphate/ml (5 mg tablets: Sigma) in 10% diethanolamine buffer (pH 9.8) were added to each well and the plate was scanned for absorbance at 405 nm (A_{405}) by an E-MAX Microelisa Reader (Molecular Devices Incorporation, Sunnyvale, CA, USA) when the absorbance of the positive reference serum had reached a level between 0.80 and 1.00 after incubation for 15–20 min. Positive reference serum consisted of the mean value of eight different pre-tested serum samples from previously pZP-immunized horses. The negative control pools were derived from 12 non-immunized random horses. The experimental sera were tested in duplicates and their results were expressed as a percentage of the positive reference serum and absorbance ratios. Percentage of the positive reference serum is calculated by subtracting the negative reference serum absorbance (NRa_{405}) value from the mean of the experimental absorbances (Ea_{405}), divided by the positive reference absorbance (PRa_{405}) value and multiplying by 100, using the formula (percentage of the positive reference serum = (Ea_{405} − NRa_{405}/PRa_{405}) × 100). Adjustments for the percentage of positive reference serum were made for each individual plate tested. The same positive and negative reference sera were used throughout the assay. Positive serum consisted of a pool of eight different pre-tested, positive serum samples and served as the reference serum. The negative control pools

were derived from 12 non-pZP-immunized random horses. Results expressed as mean absorbance ratio were determined by dividing the mean $\pm$ S.E.M. of the duplicate experimental absorbances by the mean absorbance of the reference serum tested on the same plate (Ea_{405}/rA_{405}).

Statistical analysis

The data were analyzed by one-way ANOVA. The Tukey least significant difference test was used for comparison of the means between groups.

Results

All pZP-inoculated animals responded to the preparations, as determined by the presence of anti-pZP antibodies (Fig. 1). There was a significant increase in antibody titers during the first 8 weeks in all pZP-injected animals. Anti-pZP titers, indicated as the percent of the positive reference serum, peaked at 265, 160, 125 and 105% in Group 1, Group 2, Group 3 and Group 4 respectively, and 100% of the positive reference serum in the standard two-injection protocol (Group 6). Peak titers in each group of mares were followed by a steady decline in antibody titers and remained detectable for at least 43 weeks. Of the four groups of mares which were administered various adjuvant formulations in a single injection, mares in Group 1 (400 µg pZP+FCA, one controlled-release pellet containing 200 µg pZP and another 250 µg QS-21) consistently yielded the highest antibody titer over a 42 week period (Fig. 2). The test for significance between groups was highly significant ($P < 0.00001$). The Tukey test for comparison of the means showed that all four groups inoculated with single-inoculation pZP formulations were statistically different from one another with the exception of Groups 2 and 4. The hierarchy of differences was Group 1 > 3 > 2, 4. When Groups 2, 3 and 4 were pooled together and compared with Group 1, there was a 15% higher titer ($P < 0.05$) in Group 1 (pZP+QS-21 adjuvant) compared with the pooled Groups 2, 3 and 4 (pZP+Carbopol adjuvants) (Fig. 3). The level of anti-pZP antibodies generated as a result of a single-injection protocol was lower than the level of antibodies generated as a result of administering a standard two-injection protocol (Group 6) using FCA and FIA (Fig. 1). No statistical comparisons were made with the two-injection protocol group of mares since one of the two mares in this group unexpectedly died from an abdominal colic after
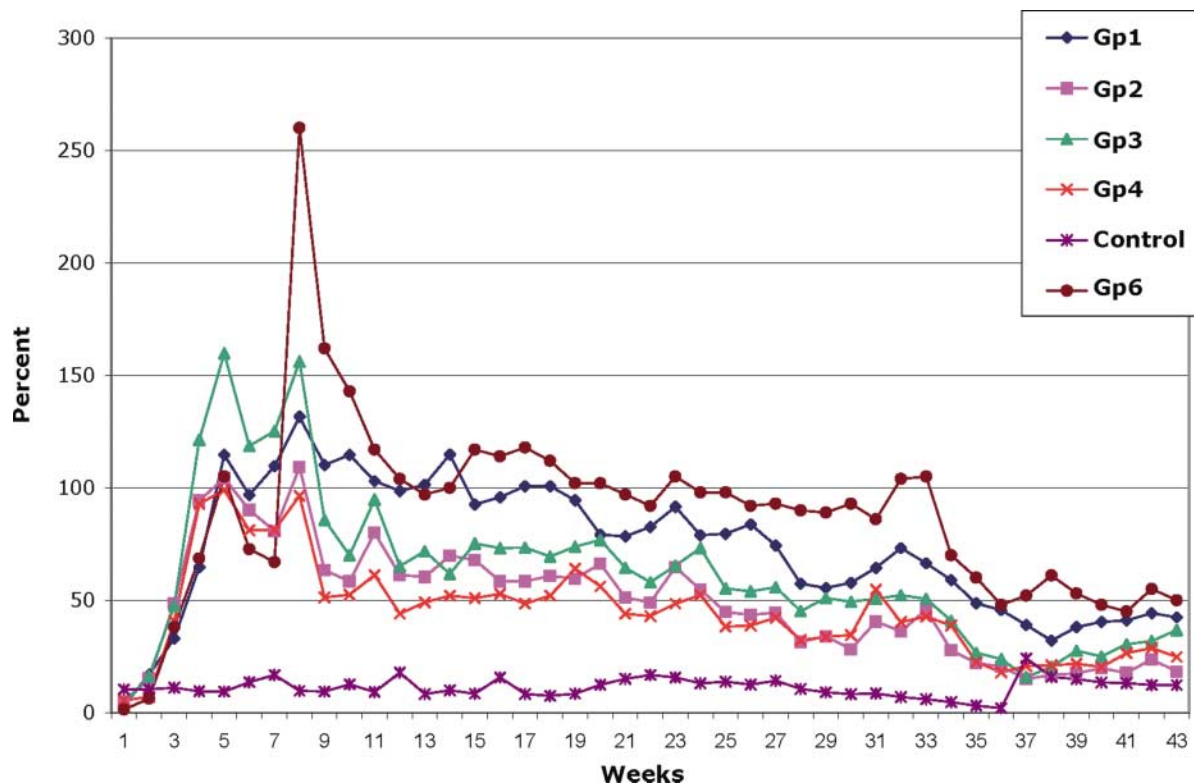

Figure 1 Anti-pZP antibody titers in samples taken every week, from week 0 to week 43 after immunization (Tx). Data are presented as group means ($\blacklozenge$), Group 1, ($n = 5$) injected with 400 µg pZP + FCA/200 µg pZP/250 µg QS-21; ($\blacksquare$), Group 2, ($n = 5$) with 400 µg pZP + FCA/7 mg Carbopol; ($\blacktriangle$), Group 3, ($n = 5$) with 400 µg pZP + FCA/200 µg pZP/7 mg Carbopol; (X), Group 4, ($n = 5$) with 65 µg pZP + FCA/200 µg pZP/7 mg Carbopol; (*), Group 5, ($n = 5$) with 1 ml PBS/250 µg QS-21; ($\bullet$), Group 6, ($n = 2$) with 65 µg pZP + FCA as first injection and 65 µg pZP + FIA as second injection. There was a significant difference between pZP-treated mares (Groups 1–4) and QS-21 adjuvant-only mares (negative control, Group 5) throughout the first 37 weeks.
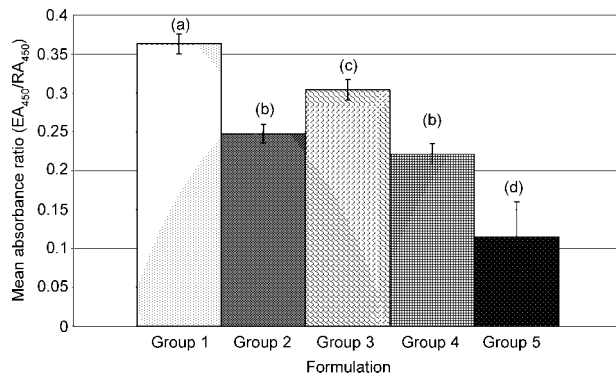
Figure 2 Forty-two week mean anti-pZP antibody titer (mean absorbance ratio) for Groups 1–5 with five mares per group. Means± S.E.M.There was a significant difference ($P < 0.00001$) in mean anti-pZP antibody titer between Group 1 mares and all other groups inoculated with pZP. No differences were found between Groups 2 and 4. Group 0 is not included (only one mare represented in this group). Statistically significant differences between values ($P < 0.05$) are indicated by different letters.

Figure 3 Comparison of anti-pZP antibody titers when either QS-21 (Group 1) or pooled Carbopol adjuvants (Groups 2–4) are used. Data are expressed as mean absorbance ± S.E.M. There was a significant difference ($P < 0.05$) in anti-pZP antibody titers between Group 1 and pooled samples of Groups 2–4.

2 months into the study. Hence, the anti-pZP antibody titers of Group 6 are represented by only one mare. No behavioral, systemic or local adverse effects were noted in any horses following the inoculation of the pZP preparations throughout the study.

Two-dimensional PAGE analysis of pZP components

Figure 4a, illustrates the silver-stained two-dimensional PAGE-separated pZP glycoproteins. Four heterogeneous components were observed of 25, 55, 65 and 90 kDa and designated 25 kDa, 55 kDa, 65 kDa and 90 kDa as per the previous observations of Hedrick & Wardrip (1987). The 55 kDa, 65 kDa and 90 kDa components appeared as a 'beads on a string' series of spots diagonally aligned, with increasing acidity accompanying an increase in molecular weight for all components. The 25 kDa component, present in smaller amounts than the other pZP components, exhibited very faint staining. Thus, the partially purified pZP used in this study contained the same glycoprotein components as determined in earlier studies. Additionally, the same homogenate of partially purified pZP was used to inoculate horses in a previous study and antibodies generated from these horses were demonstrated to significantly reduce zona binding of porcine sperm to porcine oocytes (Liu *et al.* 1989).

Western blot: mare serum immunoreactivity

Figure 4b illustrates the immunoreactivity of pooled mare sera and Fig. 4c, single mare serum with the two-dimensional PAGE-separated pZP components. All of the pZP components (25 K, 55 K, 65 K and 90 K) of the single and pooled serum samples contained epitopes for the mare serum antibodies. The 25 K component was detected as a very faint band, consistent with the silver staining results (Fig. 4a).
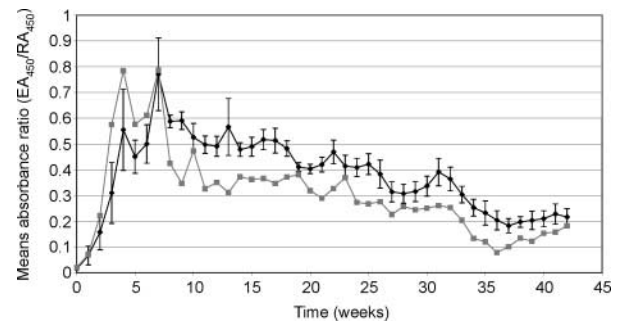
Immunohistochemistry

Horse and dog oocytes were used to determine the specificity of the anti-pZP antibodies. The patterns of staining of the zonae pellucidae, using pooled horse sera from treated horses in both horse and dog oocytes were intense (Fig. 5A and C). Immunohistochemical labeling of the zonae pellucidae with sera from untreated horses in horse and dog oocytes was not observed (Fig. 5B and D). The use of goat anti-horse IgG as the secondary antibody suggests that antibodies generated as a result of pZP inoculations were IgG. Cross-reactivity of horse anti-pZP antibody to the canine zona pellucida was evident and shown in Fig. 5C.

Hormone analyses

Progesterone analyses of all mares treated indicated a high incidence (82.6%) of persistent corpora lutea. Of the 25 mares that were inoculated with the pelleted pZP preparation, 17 (85%) exhibited evidence of persistent corpora lutea, ranging in duration from 35 to 206 days with a mean persistency of 79.3 days (Fig. 6). All five control mares (Group 5) that received PBS and pellets containing only QS-21 adjuvant, also exhibited persistent corpora lutea ranging in duration from 54 to 145 days with a mean persistency of 89.5 days. One of two mares (50%) inoculated with the two-inoculation protocol also exhibited evidence of persistent corpora lutea during the course of the investigation that lasted for 60 days. While the incidence of persistent corpora lutea was significantly elevated among the pZP-treated and control groups of mares in this study, all mares exhibiting persistent corpora lutea eventually returned to normal cyclicity following a prolonged diestrous period.

Discussion

The pZP immune response exhibited in mares using the various pZP immunization protocols in this study was against all the pZP glycoprotein components as shown by
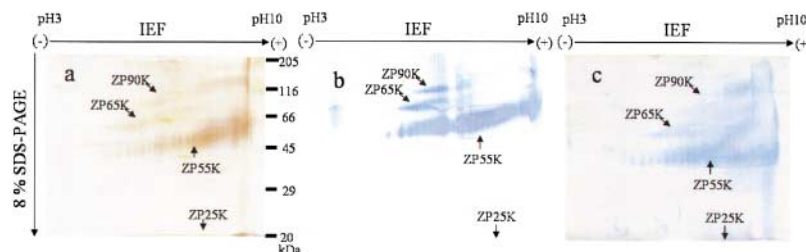
Figure 4 Two-dimensional PAGE gel pattern of pZP and the immunoreactivity of horse anti-pZP antiserum. A two-dimensional PAGE gel of pZP was stained with silver (a). The separated pZP glycoproteins on a separate gel were transblotted onto a PVDF membrane and transferred immunoreactive pZP glycoproteins were detected with pooled (b) and single (c) horse anti-pZP. The numbers in the center indicate molecular weight markers; the arrow from the left to right shows the direction of IEF separation, and the arrow from the top to the bottom shows the migration direction in 8% SDS-PAGE.

the Western blotting experiments. Our observations on the immunoreactivity of the mare serum and conclusions concerning the potential immunocontraceptive properties of the pZP are consistent with those of Kitchener *et al.* (2002). Using traditional immunization methods (FCA with booster immunizations), Kitchener *et al.* found

effective pZP immunocontraception in the tammar wallaby. They observed that all pZP components were immunoreactive with wallaby serum antibodies, consistent with the observation reported here using controlled-release immunization methods. Thus, the immunization method of choice for the mare can be determined by
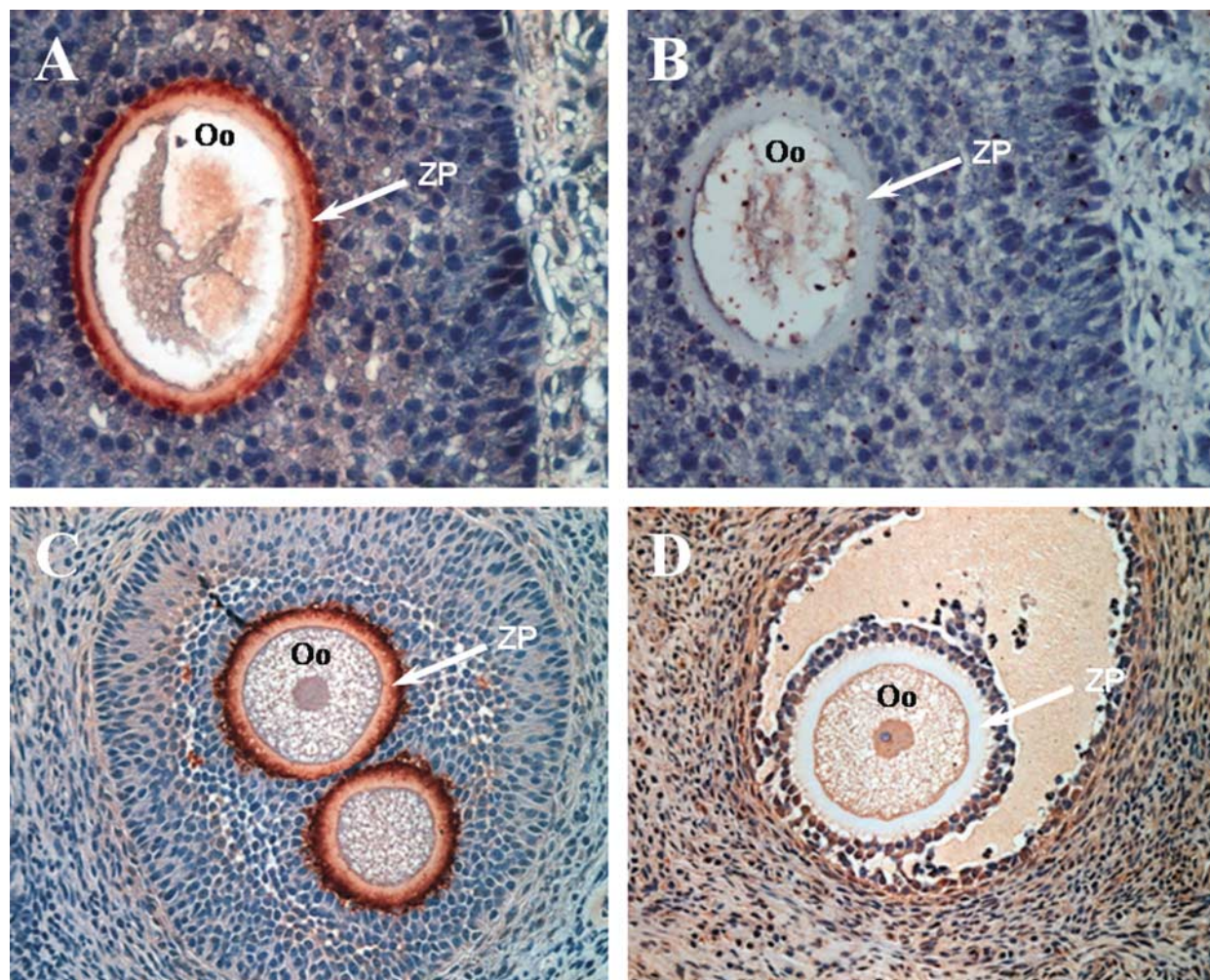

Figure 5 Immunohistochemical staining of horse follicle (10 ×) and dog ovaries (10 ×). Anti-pZP antibodies from pooled sera of pZP-treated horses bound to zonae pellucidae (ZP) (arrow) of oocyte (Oo) in horse (A) and oocyte of dog (B). Antibodies from pooled sera of untreated horses did not display zonae pellucidae staining of oocytes in horse (B) and dog (D).
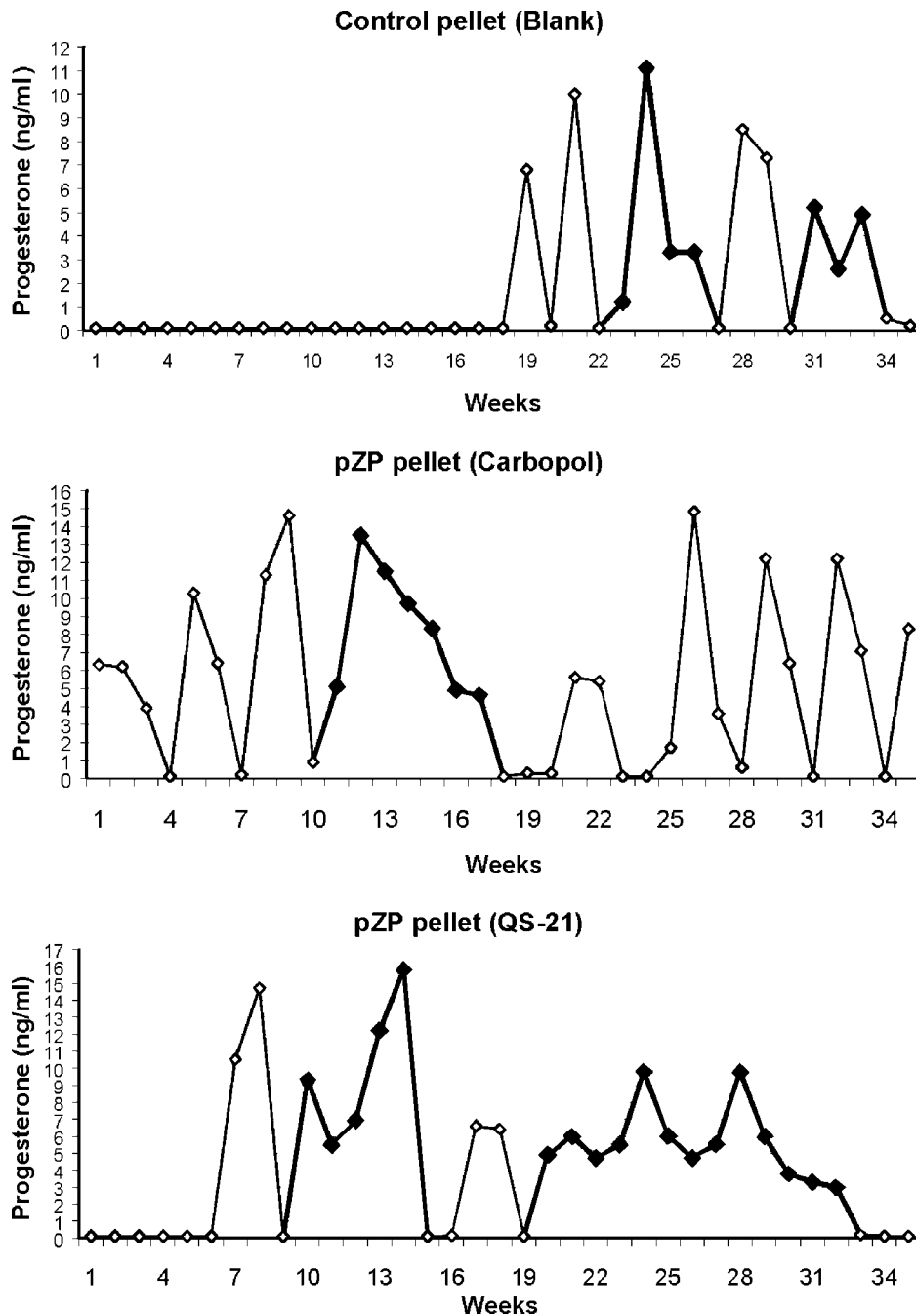
Control pellet (Blank)

pZP pellet (Carbopol)

pZP pellet (QS-21)

Figure 6 Weekly progesterone levels in three (Control, pZP + Carbopol pellet and pZP + QS-21 pellet) mares. Weeks of persistent corpus luteum indicated by ◆ and normal cycling indicated by ◇.

practical considerations, e.g. ease and cost of immunization, since the biology of the immune response and its effect on the animal's reproductive physiology does not seem to vary according to the immunization protocol.

The level of anti-pZP antibodies generated as a result of a single inoculation in this study is equivalent to those reported in other studies using multiple inoculations (Liu *et al.* 1989, Kirkpatrick *et al.* 1992, Willis *et al.* 1994). The significant increase in antibody titers within 2 months after the inoculation, and that an increased level of anti-pZP antibody was sustained for up to at least 35 weeks, indicated a release of pZP from the pellets. However, it

was not clear when the pZP incorporated in the pellet was released. There was no observable booster effect as anticipated in any of the groups treated with the pelleted pZP. This was apparent from comparison of the antibody responses of Groups 1, 3 and 4 mares with Group 2 mares (blank pellets). In spite of efforts to fabricate a controlled-release pellet that would offer a booster effect (shown by a sharp rise in anti-pZP antibody levels immediately following the presumptive release), this study failed to demonstrate such an effect. However, there was a prolonged anti-pZP antibody response consistent with those seen following repeated exposure to the antigen. It is possible that

the anticipated controlled-release of antigen was more consistent with a sustained release. Further *in vivo* and *in vitro* studies are necessary to determine the precise time and rate of pZP release when lyophilized preparations of pZP and water-soluble adjuvants are included in PLGA pellets.

Based on previous studies (Liu *et al.* 1989, Turner *et al.* 1997), there is a correlative effect of the level of antibodies generated and contraception in the mare. In these studies, anti-pZP antibodies reaching 50–60% of the positive reference serum was considered as the threshold in which 90–95% of the mares will be protected from becoming pregnant. In this study, the duration of the anti-pZP antibody threshold for 95% contraceptive effectiveness generated from Group 1 was equal to that of the two-inoculation protocol, suggesting that the single-inoculation protocol utilized in Group 1 mares may serve as an effective procedural alternative for contraception in horses. The combined formulation incorporating QS-21 adjuvant and pZP into PLGA pellets, along with an emulsified preparation of pZP+FCA, appears to be more effective for single-inoculation administration in horses than combinations incorporating pZP preparations and Carbopol as the adjuvant. Mares treated with Carbopol adjuvant only were not used in this study.

This study used equine and dog oocytes to identify specific labeling of the anti-pZP antibodies generated by the inoculated horses. Specific and strong labeling was observed on the zonae pellucidae of the equine and dog oocytes. Because cross-reactivity of porcine antibodies with dog zona pellucida have been described in other studies, we selected the dog ovary to further document the specificity of anti-pZP antibodies raised in horses against zonae pellucidae. This study is consistent with cross-reactivity investigations reported for the dog (Mahi-Brown *et al.* 1988, Barber *et al.* 2000).

Progesterone analysis was used as an indicator of cyclicity in the treated mares. There was a significantly increased incidence of prolonged corpora lutea function among the treated mares. In several instances the presence of a prolonged corpus luteum was extended beyond that period which is seen in normal mares. During the normal physiological breeding season, the incidence of persistent corpora lutea in mares ranges from 3.6 to 25% (Ginther 1992) and the normal duration of persistent corpora lutea is reported to be in the range of 35–95 days. We were unable to determine the cause for the increased incidence and duration of prolonged diestrous cycles in these treated mares, including the mare receiving two inoculations. Previous studies in mares using at least two inoculations of pZP (FCA, injection 1; FIA injection 2) have reported normal cyclicity (Liu *et al.* 1989, Kirkpatrick *et al.* 1990). Only two mares in the present investigation were administered two inoculations, thus limiting conclusions regarding corpora lutea function in this group. In view of the high incidence of persistent corpora lutea also occurring in the control group of mares that received

PLGA pellets containing QS-21 adjuvant, it is likely that there is a relationship between the inoculation of pellets and persistent corpora lutea in the mare. Further investigations are warranted to define the relationship between PLGA copolymers and persistent corpora lutea in mares. Regardless of the increased incidence and extent of the prolonged corpora lutea, all mares returned to normal cyclicity after their prolonged diestrual period (Fig. 6). This was demonstrated by decreased levels of progesterone (<1.0 ng/ml) for a period of at least 5 days followed by another rise in progesterone level, indicating resumption of estrus with subsequent ovulation.

The mares in this study were not monitored for behavioral estrus and receptivity on a routine basis nor were they mated. The objective of this study was to characterize the antibody response and the effect on the estrous cycle as a result of a single administration with controlled-release pZP formulations. Similar pZP and adjuvant formulations have been field tested in free-roaming horses in select herd management areas (Turner *et al.* 2001). Data on fecal estrone sulfate and progesterone levels and foal counts in this field study indicated 89.7% (86 of 97 mares) contraceptive effectiveness in treated mares when compared with mares not treated. However, consistent observations of matings and or sufficient sampling of feces to determine cyclicity patterns were not performed. Therefore, the high percentage of contraceptive effectiveness may have been attributable to either (i) limited matings of some treated mares due to extended diestrous periods during the physiological breeding season, (ii) normal frequency of matings without conception, or (iii) a combination thereof.

Determination of the contraceptive effectiveness in the treated mares would have made a strong contribution to this study. However, it is likely that the administration of exogenous prostaglandin would have been required to induce cyclicity for mating due to the extended diestrous periods in the treated mares. If prostaglandins were administered to induce voluntary cyclicity, the high incidence and prolonged duration of persistant corpora lutea in the treated mares would not have been observed. In several other studies using pZP as a contraceptive agent in horses, no adverse effects were reported on cyclicity of the treated mares (Liu *et al.* 1989, Kirkpatrick *et al.* 1991, 1992). Additionally, histopathological examinations of the ovaries from pZP-treated mares were performed in one of these studies and no pathological lesions were reported (Liu *et al.* 1989). Histological examinations of the ovaries of the treated mares in this study were not performed.

Because the fabrication and use of PLGA polymers as controlled-release systems require water-soluble adjuvants, Carbopol and QS-21 were selected for use in this investigation. The pZP incorporated into each pellet was anticipated to be released after 3–4 weeks in the muscle, providing a booster effect (D R Flanagan, unpublished observation). The adjuvant Carbopol, required a large number of pellets (six) to achieve the desired effect

as an adjuvant. In contrast, only one pellet of QS-21 was required to achieve the same desired effect. Although neither of the water-soluble adjuvants in this study generated anti-pZP antibody levels comparable with the combined oil-based FCA and FIA adjuvants, both formulations of pZP incorporating QS-21 or Carbopol generated anti-pZP antibody levels that persisted for at least 34 and 25 weeks respectively. In this study, the anti-pZP antibody level for the standard two-inoculation formulation (pZP+FCA and FIA) persisted for at least 35 weeks.

In conclusion, a single inoculation containing pZP and QS-21 adjuvant preparations formulated in PLGA copolymer pellets, combined with emulsified pZP+FCA can generate anti-pZP antibody levels equivalent to standard two-inoculation protocols. The use of a single-inoculation pZP formulation to achieve a desirable anti-pZP antibody level in horses eliminates the necessity of reassessing mares in the field for a second inoculation or holding horses in captivity for a second inoculation. Furthermore, a single-inoculation protocol will markedly enhance the cost-effectiveness for wild horse population management. Although the endocrine data showed the occurrence of persistent corpora lutea in 82.6% of the treated mares, no adverse effects of treatment were observed visually during the study. Fertility studies are necessary to determine the contraceptive effect of a single-inoculation protocol incorporating PLGA copolymer pellets.

Acknowledgements

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